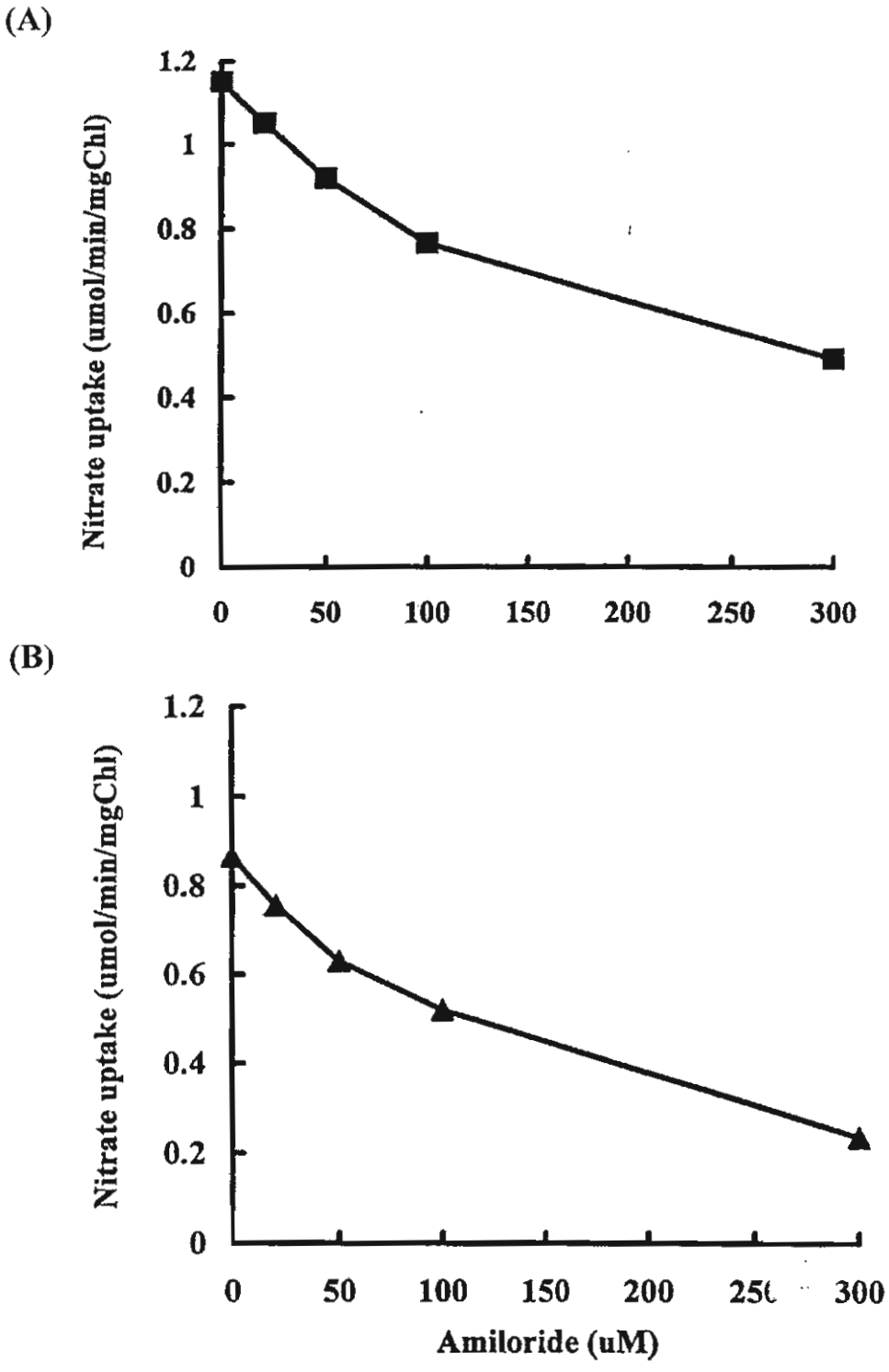


**Figure 3.16 Effect of amiloride concentration on nitrate uptake by cells grown under normal 0.5 M NaCl (A) and salt stress with 2 M NaCl (B)**

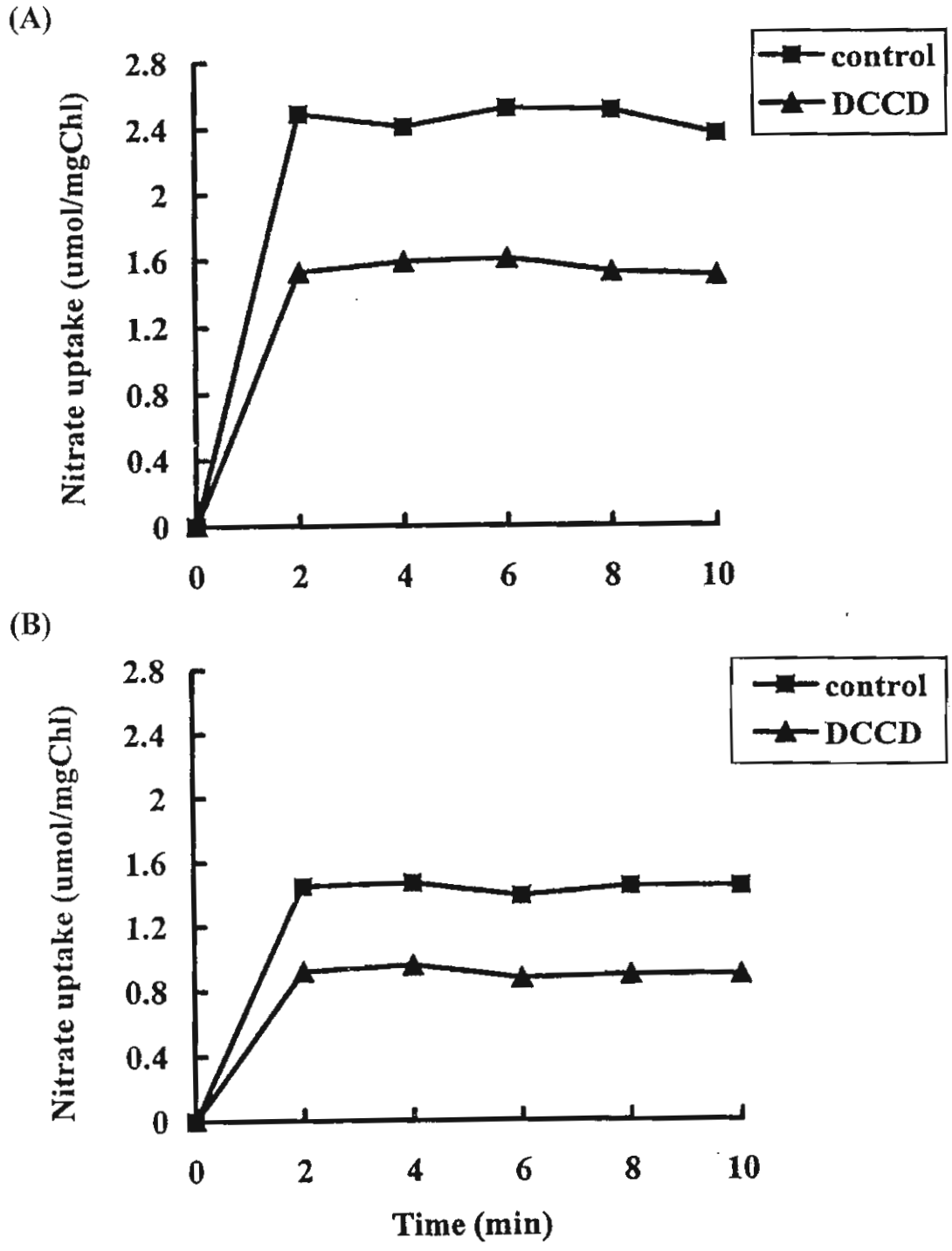


**Fig3.17 Effect of DCCD on nitrate uptake by cells grown under normal 0.5 M NaCl (A) and salt stress with 2 M NaCl (B)**

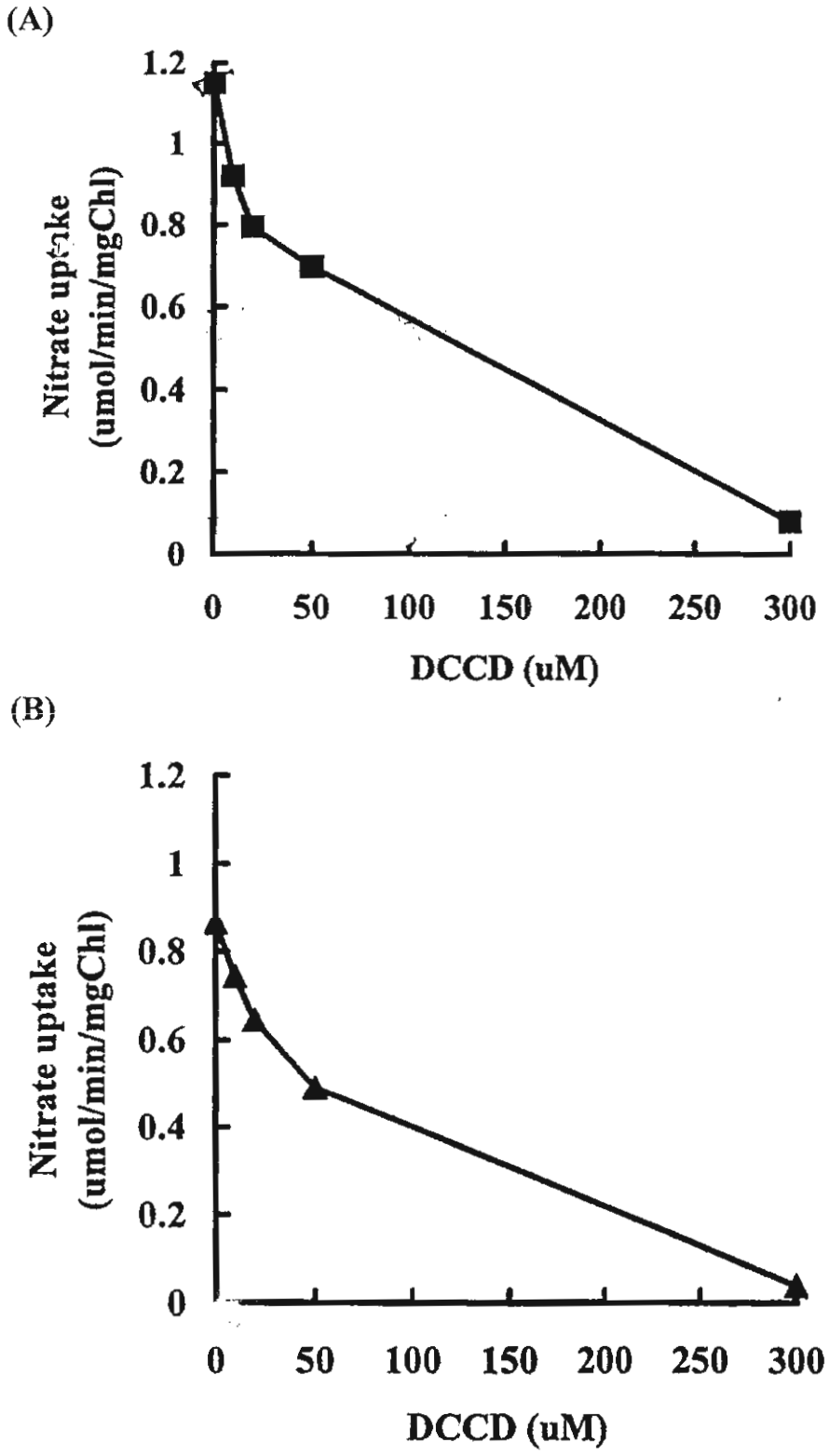


**Figure 3.18 Effect of DCCD concentration on nitrate uptake by cells grown under normal 0.5 M NaCl (A) and salt stress with 2 M NaCl (B)**

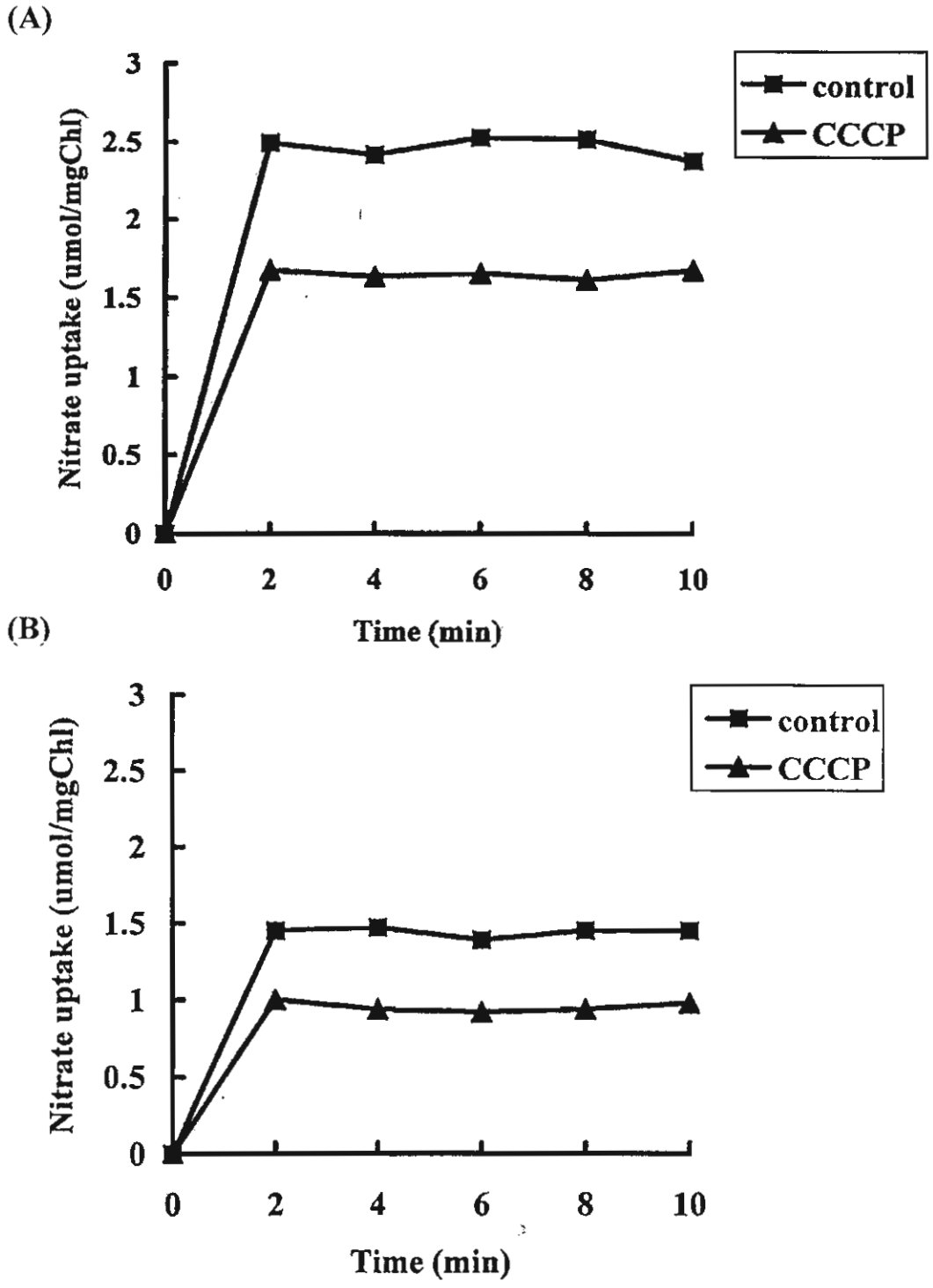


**Figure 3.19 Effect of CCCP on nitrate uptake by cells grown under normal 0.5 M NaCl (A) and salt stress with 2 M NaCl (B)**

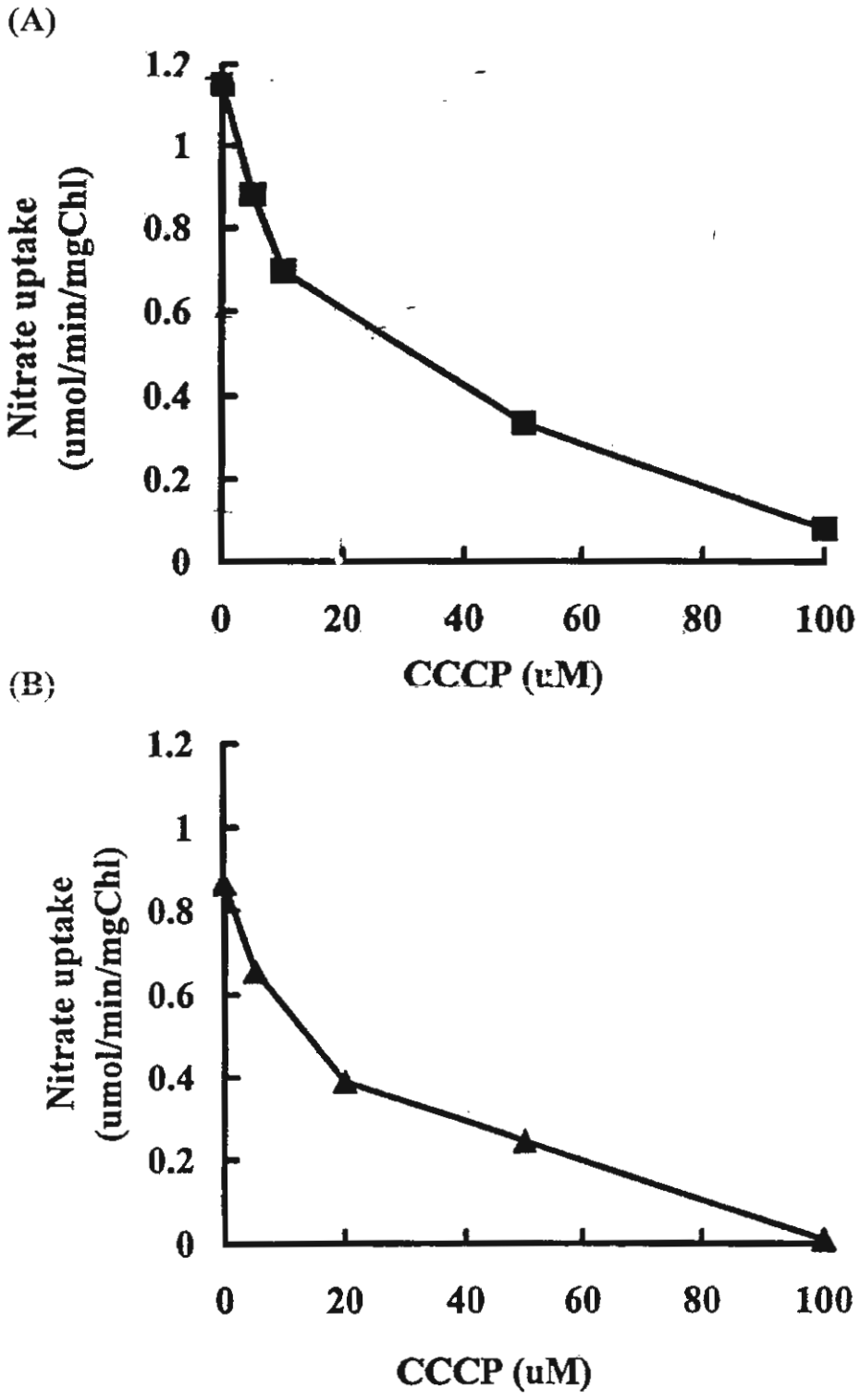


**Figure 3.20 Effect of CCCP concentration on nitrate uptake by cells grown under normal 0.5 M NaCl (A) and salt stress with 2 M NaCl (B)**

complete in the presence of 300 μM CCCP and 300 μM DCCD (Figs, 3.18, 3.20). These results seem to suggest that a pH gradient generated by $H^+$/ATPase drives nitrate uptake in *A. halophytica*.

The monensin effect indicates that nitrate uptake relies on the maintenance of a sodium electrochemical gradient across the plasmalemma, which may represent an immediate source of energy for active nitrate uptake. Amiloride treatment of *A. halophytica* resulted in a decreased rate of nitrate uptake. These data suggest that nitrate uptake in *A. halophytica* cells is partly attributed to the $Na^+/H^+$ antiporter activity.

### 3.3.3 Combined effects of metabolic inhibitors on nitrate uptake

Treatments that decrease ATP synthesis (i.e. by DCCD) and treatments dissipating the proton and $Na^+$ gradients (i.e. by CCCP and monensin) led to the reduced nitrate uptake. We therefore investigated further how each individual inhibitor of driving force when present alone or in combination would affect nitrate uptake. Figure 3.21 shows that either monensin or CCCP or DCCD could reduce nitrate uptake by about 50% when compared to that in the absence of inhibitor. When two inhibitors were present together in any combination, only about 10% of nitrate uptake activity was detected. Interestingly, the presence of three inhibitors together almost completely abolished nitrate uptake.

## 3.4 Effect of energy source on nitrate uptake

In order to test whether exogenously added energy source can influence nitrate uptake, glucose an lactate were employed for this purpose. Glucose or lactate could increase nitrate uptake to a similar extent (Fig. 3.22). When glucose and lactate were present together, there was hardly any further increase of nitrate uptake. *Aphanothece*

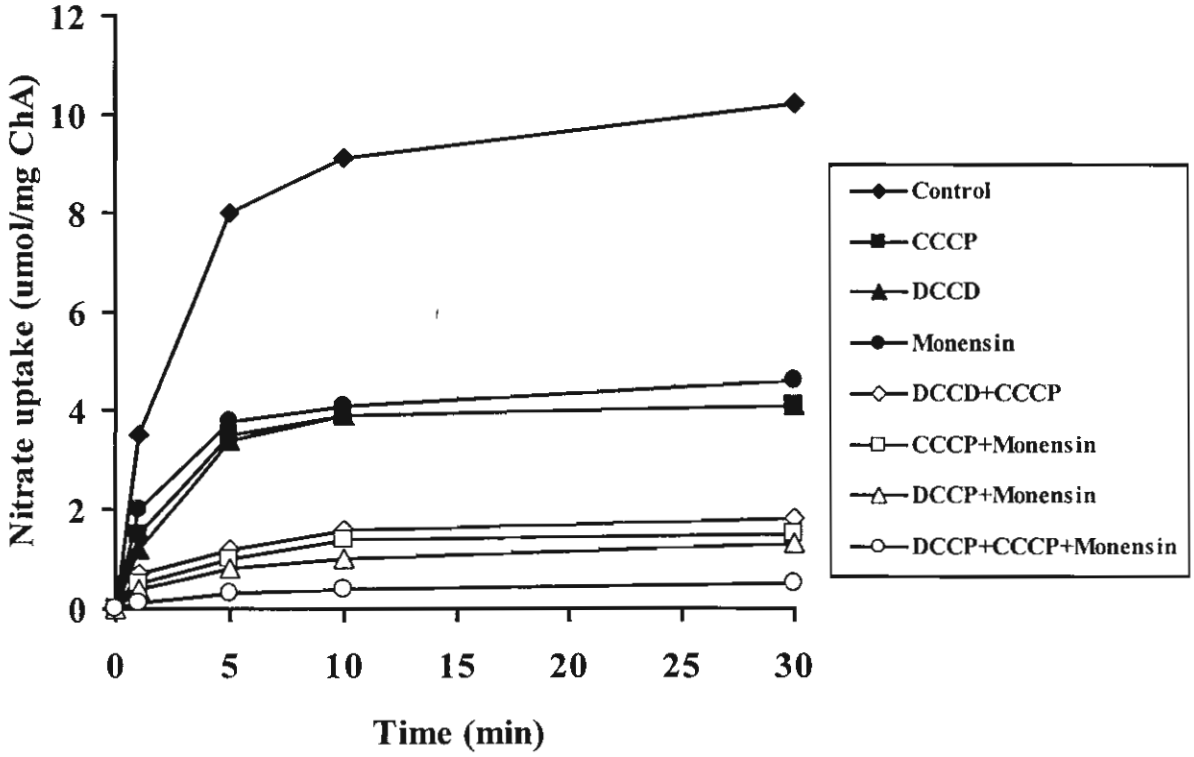


**Figure 3.21 Effect of various inhibitors on nitrate uptake by normal cells**

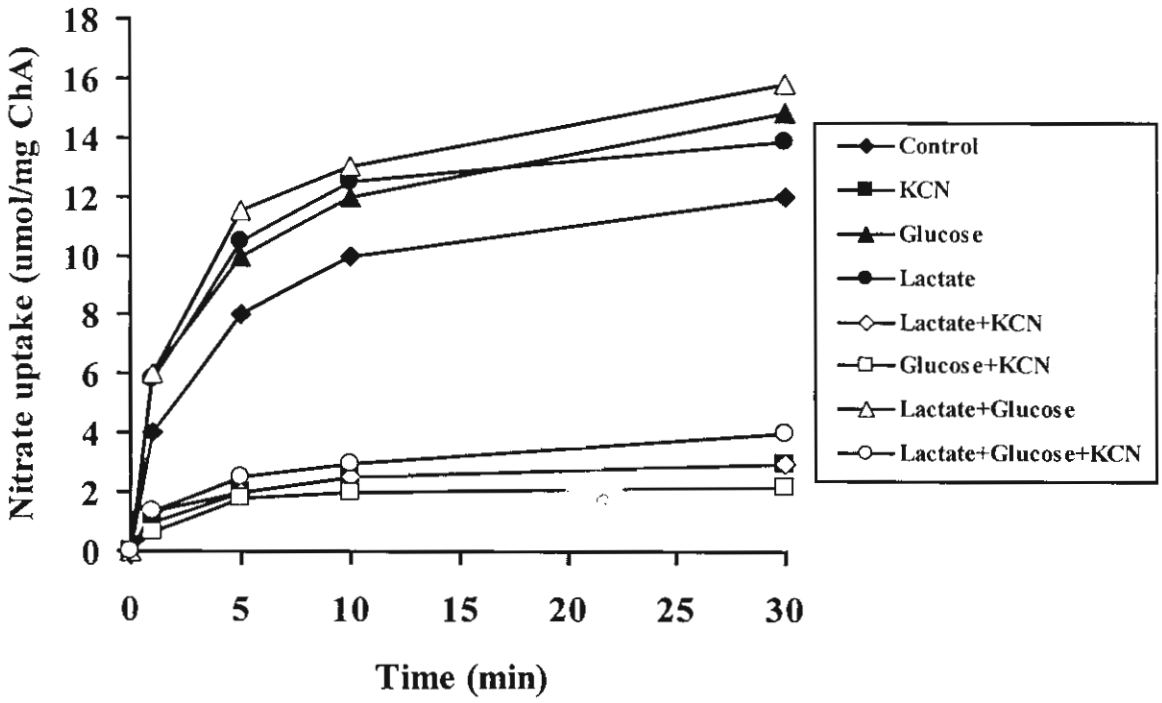


**Figure 3.22 Effect of KCN, lactate and glucose on nitrate uptake by normal cells**

*halophytica* cells without energy source supplementation exhibited considerable nitrate uptake. This was probably due to the transport driven by endogenous energy source. This contention was supported by the abolition of nitrate uptake in the presence of KCN, an inhibitor of an energy-yielding respiration. The strong inhibitory effect of KCN on nitrate uptake prevailed regardless of the presence of glucose alone or in combination with lactate. The residual nitrate uptake observed even in the presence of KCN might arise as a result of passive diffusion of nitrate into the cells.

# CHAPTER 4

# DISCUSSION

## Characterization of nitrate uptake system

The effect of salinity on nitrate uptake has been studied in the halotolerant cyanobacterium *Aphanothece halophytica* with reduced nitrate reductase activity and hence the transported nitrate nitrate inside the cells would be minimally reduced. This has been accomplished by tungstate treatment of *A. halophytica* to generate cells with reduced levels of nitrate reductase activity. Molybdenum is a prosthetic group of cyanobacterial nitrate reductase with an essential role in catalysis (Guerrero and Lara, 1987). Under conditions of molybdenum deprivation, tungsten can be incorporated into newly synthesized apoprotein in place of molybdenum, leading to the formation of an inactive nitrate reductase (Lara et al, 1987). Figure 3.1 shows that nitrate uptake in normal condition is higher than that in salt stress condition. Furthermore, the low level of nitrate uptake observed at higher concentrations of external nitrate is probably a case of passive diffusion.

When nitrate concentration was varied the nitrate uptake system followed typical Michaelis-Menten kinetics. Using Lineweaver-Burk transformation of the data, *A. halophytica* showed $K_s$ values in normal and salt stress conditions of 416 and 450 μM respectively. Similarity in $K_s$ values suggest that salt stress causes no alteration on the binding site of the nitrate transport protein. The $K_s$ values for nitrate uptake by *A. halophytica* was much hither than that reported for a filamentous cyanobacterium *Anabaena* sp. PCC 7120 (Ks = 31 μM) (Rai and Tiwari, 1999). In this respect it should be noted that in the present study 0.5 M sorbitol was present

in the assay medium of nitrate uptake to maintain the turgor pressure of *A. halophytica*. The possibility that sorbitol might hinder the binding of nitrate to the transporter cannot be ruled out. However, ***A. halophytica*** also showed a uniphasic nitrate uptake system similar to ***Anabaena*** sp. 7120.

The data presented in this study clearly showed that *A. halophytica* had a reduced uptake of nitrate when grown under salt stress (Fig. 3.1). This is in contrast with the previous report in a nitrogen-fixing *Anabaena torulosa* showing an increase nitrate uptake when grown in 170 mM NaCl (Reddy et al, 1989). The increase of nitrate uptake in *A. torulosa* was related to the increased salt tolerance of the cells via the inhibition of $Na^+$ influx which is proposed to be a major mechanism for protection of *A. torulosa* against salt stress. In the present study, log-phase *A. halophytica* grown under salt stress was used. At this phase of growth salt stress adaptation via the accumulation of glycine betaine has been complete. This was substantiated by the previous observation that the accumulation of glycine betaine already reached mixiumum after 5 days of growth in the medium containing 1.5 M NaCl (Ishitani et al, 1993). Therefore, a reduced nitrate uptake by *A. halophytica* grown under salt stress may not be related to salt stress adaptation. Another contributing factor for the salt stress protection is via the regulation of intracellular $Na^+$. Recently it has been reported that the efflux of $Na^+$ catalyzed by a $Na^+$ / $H^+$ antiporter appeared to be a major mechanism for salt stress protection of *A. halophytica* (Waditee et al, 2001 and Waditee et al, 2002).

## $Na^+$ requirement for nitrate uptake

The uptake of nitrate by *A. halophytica* was dependent on the presence of $Na^+$ as illustrated in Fig. 3.3 This finding agrees with a previous study in *Anacystis nidulans* R2 (Rodriguez et al, 1994). The study showing that monensin, a dissipator of the electrochemical potential for $Na^+$, could

inhibit nitrate uptake also supported the requirement of $Na^+$ for nitrate uptake by *A. halophytica* (Fig. 3.14). Not only is nitrate uptake dependent on $Na^+$ but the uptakes of other solutes also require $Na^+$ as coupling ions in a number of organisms, most notably in alkalophilic and halophilic bacteria (Rosen, 1986). Stimulation of the uptake of glycine betaine (Moore et al, 1987) and choline (Incharoensakdi and Karnchanatat, 2003) by $Na^+$ has also been shown in *A. halophytica*.

## Inhibitory effect of $NH_4^+$ on nitrate uptake

Previous studies on nitrate uptake by some cyanobacteria and microalgae have shown the inhibitory effect of ammonium and that the inhibition was found to be mediated by ammonium assimilation products (Omata, 1995). Our results in Fig. 3.5 also give further support for nitrate uptake inhibition by ammonium. However, it is not certain whether the inhibition of nitrate uptake by ammonium has any connection with the process of ammonium assimilation. We found little or no effect of methionine sulfoximine, an ammonium assimilation inhibitor, on the release of nitrate uptake from ammonium inhibition. There exists the possibility that the effect of ammonium on nitrate uptake observed in *A. halophytica* was through stimulation of nitrate efflux rather than inhibition of nitrate influx. A recent study in barley roots demonstrated the increased nitrate efflux upon exposure to ammonium (Kronzucker et al, 1999). Nevertheless, it is reasonable to expect that ammonium might have direct effect on the transport systems as well as effects at the level of transcription via products of ammonium assimilation. It was demonstrated many times that in the presence of both ammonium and nitrate in the medium, the ammonium is assimilated first, and only when it has gone is nitrate utilized. However there have been few reports showing preferential assimilation of nitrate; this phenomenon bas been reported e.g. for *Pandorina* and *Haematococcus*.

## Effect on $NO_2^-$ and anions on nitrate uptake

Nitrite is a particularly interesting candidate for regulation because it appears to be taken up by the same transporters as nitrate (Aslam et al, 1992). The kinetics of nitrate uptake inhibition by nitrite showing low $K_i$ value (Figs. 3.8, 3.9) suggest that the inhibition is of competitive type, i.e. both nitrate and nitrite are taken up by the same uptake system. This is in agreement with previous studies in other cyanobacteria, for example *Synechococcus* sp. PCC 7942 with a nitrate-nitrite bispecific transporter (Maeda and Omata, 1997), *Anacystis nidulans* R2 (Rodriguez et al, 1992). However, a very recent study in a filamentous, heterocystous canobacterium, Nostoc ANTH indicated the existence of separated nitrate and nitrite uptake systems (Bhattacharya et al, 2002). This was based on the results of a chlorate-resistant mutant of Nostoc ANTH which lacked nitrate uptake activity but retained nitrite uptake capacity. Nitrite-specific active transport system has also been recently reported in *Synchococcus* sp. PCC 7942 (Maeda et al, 1998) Whether *A. halophytica* contains nitrite-specific transport system remains a subject of further study.

Both chloride and phosphate ha no effect on the uptake of nitrate by *A. halophytica* (Fig. 3.10). This suggests that the reduced nitrate uptake under salt stress condition is not due to chloride ion. Rather, it may be due to the deleterious effect of high sodium ion. Another possibility of reduced nitrate uptake caused by salt stress is due to its osmotic effect rather than an ionic effect.

## Reduction of nitrate uptake by an inhibitor of $CO_2$ fixation

As the assimilation of nitrogen into protein requires both energy and organic skeleton, it is not surprising that there are major interactions between N-assimilation and photosynthetic metabolism. The assimilation of both

ammonium an nitrate is dependent on phostosynthesis, that is assimilation requires light and $CO_2$; removal of either of these prevents assimilation. To investigate the relationship between nitrate assimilation an carbon dependence in *A. halophytica*, we tested whether the interruption of $CO_2$ fixation affected nitrate uptake. It turned out that DL-glyceraldehyde which is an inhibitor of $CO_2$ fixation caused a reduction in the nitrate uptake (Fig. 3.12). This indicated the strict dependency of nitrate uptake in *A. halophytica* on active $CO_2$ fixation. This is logical since $CO_2$ fixation will provide carbon skeletons as acceptors of ammonium arising from nitrate. The same phenomenon of positive relationship between nitrate uptake an $CO_2$ fixation was also observed in *Synechococcus* sp. PCC 7942 (Rodriguez et al, 1998). Both nitrate uptake an $CO_2$ fixation require energy for the process. Although the source of energy for nitrate uptake in cyanobacteria remains unsolved, the $Na^+$-dependent active transport or the $Na^+$ / $NO_3^-$ symport system has been suggested which relies on $Na^+$ electrochemical potential as a driving force (Lara et al, 1993). This would obviate the need for the nitrate uptake process to compete for another energy source, ATP, which as a result would be utilized mainly for $CO_2$ fixation. Consequently, in terms of energy requirement, the process of nitrate uptake and $CO_2$ fixation will proceed without conflict. This indirectly reinforces the contention that nitrate uptake in cyanobacteria is $Na^+$-dependent, at least in *A. nidulans* R2 and *A. halophytica*.

## Effect of various inhibitors on nitrate uptake

In an attempt to resolve the nature of nitrate uptake in *A. halophytica* we examined its response to several metabolic inhibitors. The uncouples carbonylcyanide-*m*-chlorophenylhydrazone an the ATPase inhibitor N, N-dicyclohexylcarbodiimide each severely inhibited nitrate uptake in *A. halophytica* in both normal and salt stress conditions (Figs. 3.18, 3.20)

These results suggest that a pH gradient generated by $H^+$/ATPase drives nitrate uptake in *A. halophytica*.

The plasma membranes of various prokaryotes have $Na^+/H^+$ antiproter activity. We could not ignore the possibility that the uptake of nitrate into the cells was due to a $Na^+/H^+$ antiport driven by a pH gradient. Amiloride has been used as an inhibitor of the $Na^+/H^+$ antiport and/or $Na^+$ channel blacker (at different concentration), in a wide variety of eukaryotic systems (Krulwich, 1983). Amiloride treatment of *A. halophytica* cells resulted in a decreased rate of nitrate uptake (Fig. 3.16). These data suggest that nitrate uptake in *A. halophytica* cells is caused by the $Na^+/H^+$ antiporter. However, there have been reports showing that nitrate transport in *Anacystis* is stimulated by amiloride (at pH 8) consistent with inhibition of a $Na^+$-importing/$H^+$-extruding antiport (Lara et al, 1993 and Kaplan et al, 1989). Increased $Na^+$-dependent nitrate transport activity in amiloride-treated cells indicates that the electrochemical gradient of $Na^+$ required for nitrate transport is not generated by the $Na^+/H^+$ antiport, but by other means, the sodium circuit may thus be the primary chemiosmotic event in cyanobacteria plasma membranes, at least in cells or species grown at or adapted to alkaline pH (Miller et al, 1984, Brown et al, 1990, Ritchie, 1992).

Nitrate uptake in *A. halophytica* is sensitive to monensin, an ionophore that collapses the electrochemical gradient for sodium, the inhibition being higher than 50% for monensin concentration as low as 30 μM (Fig. 3.14). These observations indicate that nitrate uptake in *A. halophytica* relies on the maintenance of an electrochemical gradient of $Na^+$ across the plasma membrane, which might represent the immediate source for active nitrate transport. Monensin is a carboxylic polyether ionophore that, in artificial system, causes the electroneutral exchange of $Na^+$ for $H^+$ and thereby collapses the $Na^+$ gradient between the cells and the medium (Pressman, 1976). *Synechococcus* cells also actively extrude $Na^+$ through an $Na^+/H^+$

antiporter (Blumwald et al, 1984) or a primary $Na^+$ pump (Brown et al, 1990). The active extrusion of $Na^+$ creates an electrochemical potential for $Na^+$ (Ritchie, 1992). The energy conserved in an electrochemical potential for $Na^+$ may, therefore, serve as an energy source for the secondary active transport of anions. The $Na^+$ electrochemical potential comprises an electrical component and a chemical component. The inhibitory effects of monensin and amiloride, which dissipate a chemical component, on nitrate uptake in *A. halophytica* suggest that at least this component of the electrochemical potential of $Na^+$ plays a role in this transport process. Indeed, an $Na^+/H^+$ antiporter has been reported to play a role in the extrusion of $Na^+$ which is one of the adaptive mechanisms for salt tolerance in *A. halophytica* (Waditee et al, 2001).

## Energy source of nitrate uptake

It appeared evident from the results showing the inhibitory effect by DCCD, CCCP and monensin that both ATP and electrochemical potential contribute to the driving force for nitrate uptake (Fig, 3.21). Another line of supporting evidence for this notion came from the experiments using glucose (which can generate ATP by respiration) and lactate (which can feed electrons to the electron transport chain, hence producing proton potential across the membrane), both of which could increase nitrate uptake (Fig. 3.22).

In summary, overall results in this project indicate that *A. halophytica* showed the difference in $V_{max}$ of nitrate uptake between non-stress and salt-stress cells. Other parameters tested did not elicit different responses between the two cell types. Under salt stress *A. halophytica*, in a short term response, would utilize $Na^+$ / $H^+$ antiporter to extrude $Na^+$ out of the cytoplasm in exchange for $H^+$ (Waditee et al, 2001 and Waditee et al, 2002). However, in a long term response to salt stress the uptake of nitrate should

proceed normally. The synthesis and accumulation of an osmolyte glycine betaine can then finally be responsible for salt stress protection in *A. halophytica*. With regard to an energetic aspect, nitrate transport by *A. halophytica* relies on the driving force derived from ATP and from electrochemical potential involving $Na^+$ and $H^+$ gradients.

## Suggestions for further work

The present study has shown that in *A. halophytica* salt stress could reduce the uptake of nitrate by about one-half compared to that without salt stress. The reduced uptake could be due to the consequence of the adaptation of cells in response to salt stress by extruding $Na^+$ out of the cells via the function of $Na^+/H^+$ antiporter. In view of the fact that the uptake of nitrate requires $Na^+$ as a coupling ion and the tendency by the cells to extrude $Na^+$ under salt stress, the reduced nitrate uptake is not unexpected. The next step is to explore how the nitrate after entry into the cells can be assimilated further. Specifically, we should investigate how salt stress affects the function of nitrate reductase, an enzyme that catalyzes the formation of nitrite from nitrate. The information obtained will pave way for a better understanding of nitrogen metabolism in *A. halophytica* under salt stress.

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# Appendix 1

## Growth Medium for *A. halophytica*

1). Turks Island Salt Solution

A solution of 5 l (made with distilled water) of Turks Island Salt Solution consists of the following components :

| | |
|---|---|
| A). KCl | 3.33 g |
| B). $MgCl_2 . 6H_2O$ | 2.75 g |
| C). $CaCl_2 . 2H_2O$ | 7.33 g |
| D). $MgSO_4 . 7H_2O$ | 34.70 g |
| E). NaCl | 140.80 g |

2). BG 11 plus $NO_3$ solution

The components of BG 11 plus $NO_3$ solution are as follows :

| | |
|---|---|
| A). $NaNO_3$ | 150.0 g/l |
| B). $KH_2PO_4$ | 4.0 g/l |
| C). $MgSO_4 . 7H_2O$ | 75.0 g/l |
| D). $CaCl_2 . 7H_2O$ | 36.0 g/l |
| E). $Na_2CO_3$ | 20.0 g/l |
| F). Citric acid | 6.0 g/l |
| G). EDTA . $Na_2$ | 1.0 g/l |
| H). $FeSO_4 . 7H_2O$ | 6.0 g/l |

I). Trace element $A_5$ solution consisting of the following components in 1 l solution

| | |
|---|---|
| $H_3BO_3$ | 2.68 g |
| $MlnCl_2 . 4H_2O$ | 1.81 g |
| ZnSO4 . $7H_2O$ | 0.22 g |
| $Na_2 MoO_4 . 2H_2O$ | 0.39 g |
| CuSO4 . $5H_2O$ | 0.079 g |

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$Co(NO_3)_2 . 6H_2O$ 0.049 g

The growth medium is prepared by adding 50 ml of item 2.A and 5 ml each of items 2.B to 2.I to 5 l of Turks Island Salt Solution.

# Output

## 1. International Publications

1.1 Incharoensakdi, A., Wangsupa J. Nitrate uptake by the halotolerant cyanobacterium *Aphanothece halophytica* grown under non-stress and salt-stress conditions. Current Microbiology (2003) (in press)

1.2 Incharoensakdi, A., Karnchanatat A. Salt stress enhances choline uptake in the halotolerant cyanobacterium *Aphanothece halophytica*. Biochimica et Biophysica Acta (2003) 1621 : 102-109

1.3 Incharoensakdi, A., Laloknam, S. Nitrate uptake in *Aphanothece halophytica* is driven by both electrochemical potential and ATP. (To be submitted to Current Microbiology)

## 2. Scientific Meetings (as an invited speaker)

2.1 Glycine betaine accumulation and $Na^+/H^+$ antiporter are responsible for salt talerance in a halotolerant cyanobacterium *Aphanothece halophytica*. Presented for Protein Research Network Symposium 2002 on Protein Structure and Molecular Enzymology, at Faculty of Science, Mahidol University, 29-30 August, 2002.

2.2 Mechanism of salinity tolerance in cyanobacteria : regulation via osmoprotectant and ion homeostasis. Presented for the first National Conference on Algae and Plankton, at Kasetsart University, 20-21 March, 2003.

## 3. Award Received

3.1 Recipient of the research award from the National Research Council of Thailand for the year 2002 (Section : chemical and pharmaceutical science)

# Addendum

Two in-press papers are herewith enclosed :

1. Current Microbiology (2003)
2. Biochimica et Biophysica Acta (2003) 1621 : 102-109

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# Nitrate Uptake by the Halotolerant Cyanobacterium *Aphanothece halophytica* Grown under Non-Stress and Salt-Stress Conditions

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**Abstract.** We have compared the characteristics of nitrate uptake by *Aphanothece halophytica* grown under non-stress and salt-stress conditions. Both cell types showed essentially similar patterns of nitrate uptake toward ammonium, nitrite, and DL-glyceraldehyde. Although the affinities of nitrate to non-stress cells and salt-stress cells were not significantly different, i.e., $K_s$ = 416 and 450 μM respectively, the $V_{max}$ value for non-stress cells was about twofold of that for salt-stress cells (9.1 vs 5.3 μmol $min^{-1}$ $mg^{-1}$ Chl). Nitrate uptake by *A. halophytica* was found to be dependent on $Na^+$. Ammonium inhibited nitrate uptake, and the presence of methionine sulfoximine could not release the inhibition by ammonium. Nitrite appeared to competitively inhibit nitrate uptake with a $K_i$ value of 84 μM. Both chloride and phosphate anions did not affect nitrate uptake. DL-Glyceraldehyde, an inhibitor of $CO_2$ fixation, caused a reduction in the uptake of nitrate.

Nitrate is an essential nitrogen source for growth in many heterotrophic and photosynthetic organisms including cyanobacteria. The rate-limiting step of nitrate assimilation is its active transport into the cells prior to its reduction to ammonium by the sequential action of nitrate reductase and nitrite reductase [2]. The resulting ammonium is further incorporated into the amide nitrogen of glutamine, thus initiating amino acid biosynthesis.

Our laboratory has been using the halotolerant cyanobacterium *Aphanothece halophytica* for the study of the mechanism of salt tolerance. We found that carbon assimilation in terms of photosynthetic $CO_2$ fixation increased in *A. halophytica* under salt stress [20]. On the other hand, salt stress also caused an increase in glycine betaine, a quaternary nitrogenous compound, in *A. halophytica* subjected to salt stress [4]. So far there have been very few studies in connection with the relationship of salt tolerance to nitrate assimilation in cyanobacteria. Previously it was reported that *Anabaena torulosa* cultures exposed to salt stress showed an increased rate of nitrate uptake [15].

In the present work, we compared the characteristics of nitrate uptake in *A. halophytica* grown under non-stress and salt-stress conditions. We found that *A. halophytica* grown under salt-stress conditions exhibited a reduction in nitrate uptake rate compared with that under non-stress condition.

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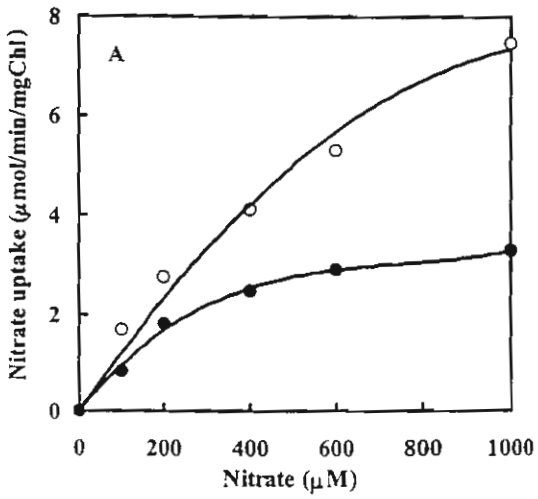
## Materials and Methods

**Organism and culture.** *Aphanothece halophytica* was grown photoautotrophically in BG 11 medium supplemented with 18 mM $NaNO_3$ as described previously [3]. A slight modification was made in which molybdenum in the medium was replaced with tungsten to induce the cells to synthesize nitrate reductase in an inactive form [7]. Cells were grown in a 250-ml flask containing 100 ml medium on a rotary shaker at 30°C without $CO_2$ supplementation. Continuous illumination was provided by cool white fluorescent lamps at an irradiance of 60 $\mu Em^{-2}$ $s^{-1}$. The concentration of NaCl was adjusted to 0.5 M for non-stress cells and 2.0 M for salt-stress cells.

**Assay of nitrate uptake.** Log-phase cells were washed with 25 mM Hepes-KOH buffer, pH 8.3, containing 12 mM $NaHCO_3$ and 0.5 M sorbitol, and were suspended in the same buffer at a chlorophyll (Chl) concentration of 25 μg/mL determined as described by Mackinney [9]. The reaction was started by the addition of 100 μM $NaNO_3$ or $KNO_3$ to the suspension kept at 30°C in the light with an irradiance of 60 $\mu Em^{-2}$ $s^{-1}$. After 2 min of incubation, the duration in which nitrate uptake increased linearly, the cell suspension was rapidly filtered through a 0.45-μm membrane filter. The nitrate content remaining in the filtrate was determined by anion-exchange high performance liquid chromatography (Hypersil-10 Sax column, 250 mm × 4.6 mm). The values shown in the figures represent the averages of two independent experiments whose variations were less than 15% of the average.



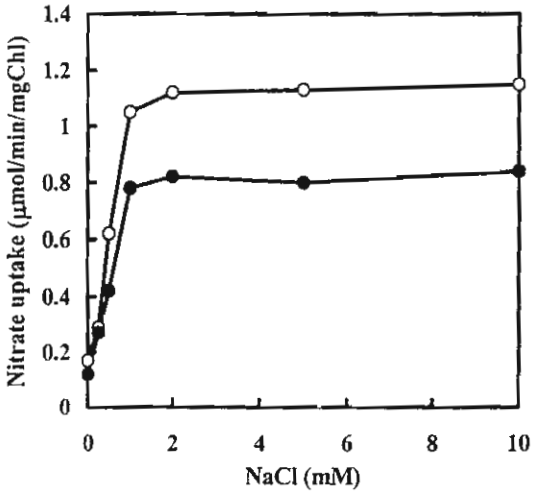




Fig. 2. Initial rates of nitrate uptake as a function of sodium concentrations by non-stress (○) and salt-stress (●) cells. The assay of nitrate uptake was done as described in Materials and Methods, but with no addition of 12 mM $NaHCO_3$, and $KNO_3$ was used instead of $NaNO_3$ in the assay medium.

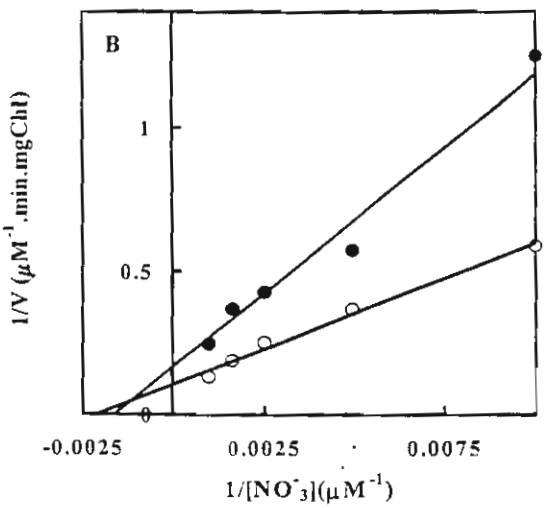



Fig. 1. Kinetics of nitrate uptake by *A. halophytica* grown under non-stress (○) and salt-stress (●) conditions. (**A**) Initial rates of nitrate uptake as a function of external nitrate concentration. (**B**) Lineweaver-Burk plots of the initial rates of nitrate uptake. The lines drawn are those derived from regression analysis of the data.

## Results

**Kinetics of nitrate uptake.** The rates of nitrate uptake by *A. halophytica* as a function of external nitrate concentration are shown in Fig. 1A. Nitrate uptake rates increased with increasing concentrations of nitrate for both non-stress and salt-stress cells. Cells under salt stress showed a lower nitrate uptake rate than those under non-stress at all external nitrate concentrations. The kinetics of uptake for both cells were of Michaelis-Menten types. The apparent $K_s$ values determined from the Lineweaver-Burk plots (Fig. 1B) for non-stress and salt-stress cells were 416 and 450 μM respectively, whereas the maximal velocities ($V_{max}$) were 9.1 and 5.3 μmol $min^{-1}$ $mg^{-1}$ Chl, respectively.

Next, we tested whether nitrate uptake was dependent on the presence of $Na^+$. Figure 2 shows that $Na^+$ could activate nitrate uptake in both non-stress and salt-stress cells. At a fixed concentration of nitrate used (100 μM), the uptake of nitrate appeared to reach saturation at 1 mM $Na^+$ for both cell types. Furthermore, nitrate uptake was not inhibited by $Na^+$ as high as 10 mM.

**Inhibition of nitrate uptake by ammonium.** The presence of higher than 100 μM ammonium in the assay medium containing 100 μM nitrate led to a reduction of nitrate uptake rate for non-stress cells, whereas for salt-stress cells inhibition of nitrate uptake was observed at higher than 50 μM ammonium (Fig. 3). Ammonium was slightly more effective for the inhibition of nitrate uptake in the salt-stress cells than in the non-stress-cells, i.e., about one-half of nitrate uptake was inhibited in salt-stress cells, whereas about one-third was inhibited in non-stress cells at 200 μM ammonium. The inhibition of nitrate uptake was nearly complete at 300 μM ammonium in both cell types. We also tested the effect of methionine sulfoximine on the prevention of ammonium inhibition of nitrate uptake. It was found that preincubation of the cells with 1 mM methionine sulfoximine did not release

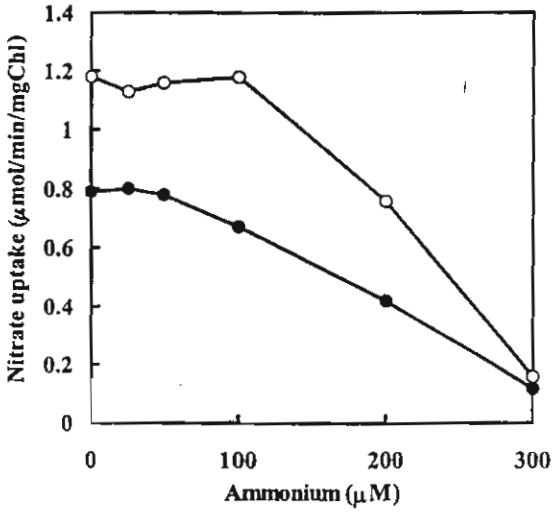



Fig. 3. Initial rates of nitrate uptake as a function of ammonium concentrations by non-stress (○) and salt-stress (●) cells.

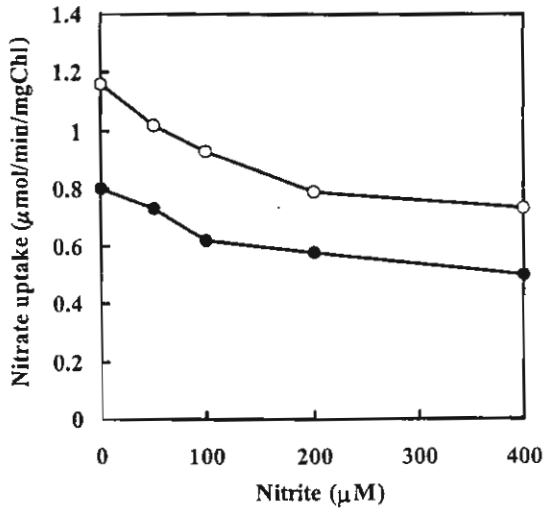



Fig. 4. Initial rates of nitrate uptake as a function of nitrite concentrations by non-stress (○) and salt-stress (●) cells.

the inhibition of nitrate uptake by 300 μM ammonium for both non-stress and salt-stress cells (data not shown).

**Effect of anions on nitrate uptake.** Nitrite could inhibit nitrate uptake in both non-stress and salt-stress cells (Fig. 4). About one-third of nitrate uptake was inhibited by 400 μM nitrite in both cells. Increasing concentration of nitrite resulted in a gradual decline in the nitrate uptake. By measuring the nitrate uptake rate at various nitrate and nitrite concentrations, the plot between inhibitor concentrations and the slopes obtained from double reciprocal plots yielded the inhibition constant ($K_i$) of 84 μM. This indicates that nitrite binds to the nitrate transporter with higher affinity than does nitrate. We also

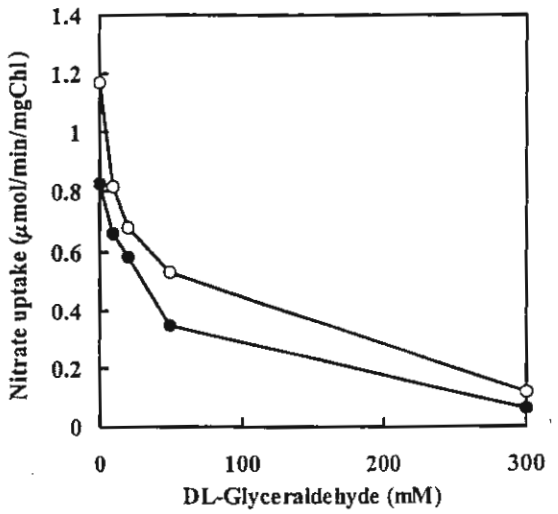



Fig. 5. Initial rates of nitrate uptake as a function of DL-glyceraldehyde concentrations by non-stress (○) and salt-stress (●) cells. The reaction mixture without nitrate was preincubated with DL-glyceraldehyde in the dark for 30 min before the addition of 100 μM $NaNO_3$ to initiate nitrate uptake.

tested the effect of chloride and phosphate anions on nitrate uptake, and the results showed that these two anions at equimolar concentration with nitrate were without effect for both non-stress and salt-stress cells (data not shown).

**Effect of $CO_2$ fixation inhibitor on nitrate uptake.** Preincubation of the cells with DL-glyceraldehyde, a selective inhibitor of $CO_2$ fixation, before nitrate uptake assay caused a reduction in the rate of nitrate uptake for both non-stress and salt-stress cells (Fig. 5). Nitrate uptake was almost completely inhibited when cells were preincubated with 300 μM DL-glyceraldehyde. The results indicated that the interruption of $CO_2$ fixation, which led to the deprivation of carbon, would inactivate nitrate transporter. Hence, it is likely that nitrate uptake [illegible] *halophytica* is highly dependent on active carbon assimilation.

## Discussion

The data presented in this study clearly showed that *A. halophytica* had a reduced uptake of nitrate when grown under salt stress. This is in contrast with the previous report in a nitrogen-fixing *Anabaena torulosa*, showing an increased nitrate uptake when grown in 170 mM NaCl [15]. The increase of nitrate uptake in *A. torulosa* was related to the increased salt tolerance of the cells via the inhibition of $Na^+$ influx, which is proposed to be a major mechanism for protection of *A. torulosa* against salt stress. In the present study, log-phase *A. halophytica*

grown under salt stress was used. At this phase of growth, salt stress adaptation via the accumulation of glycine betaine has been complete. This was substantiated by the previous observation that the accumulation of glycine betaine already reached maximum after 5 days of growth in the medium containing 1.5 M NaCl [5]. Therefore, a reduced nitrate uptake by *A. halophytica* grown under salt stress may not be related to salt stress adaptation. Another contributing factor for the salt stress protection is via the regulation of intracellular $Na^+$. Recently, it has been reported that the efflux of $Na^+$ catalyzed by a $Na^+/H^+$ antiporter appeared to be a major mechanism for salt stress protection of *A. halophytica* [21, 22].

Half saturation ($K_s$) of nitrate uptake was very similar for both *A. halophytica* grown under non-stress and salt-stress conditions, i.e., 416 μM for the former and 450 μM for the latter. This suggests that salt stress causes no alteration on the binding site of the nitrate transport protein. The $K_s$ value for nitrate uptake by *A. halophytica* was much higher than that reported for a filamentous cyanobacterium *Anabaena* sp. PCC 7120 ($K_s$ = 31 μM) [14]. In this respect, it should be noted that in the present study 0.5 M sorbitol was present in the assay medium of nitrate uptake to maintain the turgor pressure of *A. halophytica*. The possibility that sorbitol might hinder the binding of nitrate to the transporter cannot be ruled out. However, *A. halophytica* also showed a uniphasic nitrate uptake system similar to that of *Anabaena* sp. 7120.

Previous studies on nitrate uptake by some cyanobacteria and microalgae have shown the inhibitory effect of ammonium and that the inhibition was found to be mediated by ammonium assimilation products [13]. Our results in Fig. 3 also give further support for nitrate uptake inhibition by ammonium. However, it is not certain whether the inhibition of nitrate uptake by ammonium has any connection with the process of ammonium assimilation. We found no effect of methionine sulfoximine, an ammonium assimilation inhibitor, on the release of nitrate uptake from ammonium inhibition (data not shown). There exists the possibility that the effect of ammonium on nitrate uptake observed in *A. halophytica* was through stimulation of nitrate efflux rather than inhibition of nitrate influx. A recent study in barley roots demonstrated increased nitrate efflux upon exposure to ammonium [6].

The uptake of nitrate by *A. halophytica* was dependent on the presence of $Na^+$, as illustrated in Fig. 2. This finding agrees with a previous study in *Anacystis nidulans* R2 [17]. A preliminary study using monensin, a dissipator of the electrochemical potential for $Na^+$, also supported the requirement of $Na^+$ for nitrate uptake by *A. halophytica* (unpublished results). Not only is nitrate uptake dependent on $Na^+$, but the uptakes of other solutes also require $Na^+$ as coupling ions in a number of organisms, most notably in alkalophilic and halophilic bacteria [19]. Stimulation of glycine betaine uptake by $Na^+$ has also been shown in *A. halophytica* [12].

The kinetics of nitrate uptake inhibition by nitrite showing low $K_i$ value suggests that the inhibition is of a competitive type, i.e., both nitrate and nitrite are taken up by the same uptake system. This is in agreement with previous studies in other cyanobacteria; for example, *Synechococcus* sp. PCC 7942 with a nitrate–nitrite bispecific transporter [10], *Anacystis nidulans* R2 [16]. However, a very recent study in a filamentous, heterocystous cyanobacterium, *Nostoc* ANTH, indicated the existence of separate nitrate and nitrite uptake systems [1]. This was based on the results of a chlorate-resistant mutant of *Nostoc* ANTH, which lacked nitrate uptake activity but retained nitrite uptake capacity. A nitrite-specific active transport system has also been reported recently in *Synechococcus* sp. PCC 7942 [11]. Whether *A. halophytica* contains a nitrite-specific transport system remains a subject of further study.

To investigate the relationship between nitrate assimilation and carbon dependence in *A. halophytica*, we tested whether the interruption of $CO_2$ fixation affected nitrate uptake. It turned out that DL-glyceraldehyde, which is an inhibitor of $CO_2$ fixation, caused a reduction in the nitrate uptake (Fig. 5). This indicated the strict dependency of nitrate uptake in *A. halophytica* on active $CO_2$ fixation. This is logical, since $CO_2$ fixation will provide carbon skeletons as acceptors of ammonium arising from nitrate. The same phenomenon of a positive relationship between nitrate uptake and $CO_2$ fixation was also observed in *Synechococcus* sp. PCC 7942 [18]. Both nitrate uptake and $CO_2$ fixation require energy for the process. Although the source of energy for nitrate uptake in cyanobacteria remains unsolved, the $Na^+$-dependent active transport or the $Na^+/NO_3^-$ symport system has been suggested which relies on $Na^+$ electrochemical potential as a driving force [8]. This would obviate the need for the nitrate uptake process to compete for another energy source, ATP, which as a result would be utilized mainly for $CO_2$ fixation. Consequently, in terms of energy requirement, the process of nitrate uptake and $CO_2$ fixation will proceed without conflict. This indirectly reinforces the contention that nitrate uptake in cyanobacteria is $Na^+$ dependent, at least in *A. nidulans* R2 and *A. halophytica*.

Overall results in the present study indicate that *A. halophytica* showed a difference in $V_{max}$ of nitrate uptake between non-stress and salt-stress cells. Other parameters tested did not elicit different responses between the two cell types. Under salt stress, *A. halophytica*, in a

short-term response, would utilize $Na^+/H^+$ antiporter to extrude $Na^+$ out of the cytoplasm in exchange for $H^+$ [21, 22]. Since the uptake of nitrate appeared to require $Na^+$ as a coupling ion, the reduced nitrate uptake under salt stress seen in Fig. 1 was not unexpected. However, in a long-term response to salt stress, the uptake of nitrate should proceed normally. The synthesis and accumulation of an osmolyte glycine betaine can then finally be responsible for salt-stress protection in *A. halophytica.*

ACKNOWLEDGMENTS

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# Salt stress enhances choline uptake in the halotolerant cyanobacterium *Aphanothece halophytica*

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**Abstract**

The uptake of [$^{14}$C]choline by a suspension of exponential-phase *Aphanothece halophytica* under various conditions has been studied. Salt stress was found to enhance the uptake of choline. The kinetics of choline transport followed the Michaelis–Menten relationship with apparent $K_m$ values of 272 and 286 μM, maximum rates of transport ($V_{max}$) of 18 and 37 nmol/min/mg protein for unstressed and salt-stressed cells, respectively. Choline uptake under salt stress was significantly reduced in chloramphenicol-treated cells, suggesting that the activation by salt stress occurred via an inducible transport system. This was corroborated by the existence of the periplasmic choline binding protein, whose content was higher in cells grown under salt-stress condition. Exogenously provided choline significantly increased the growth rate of cells grown under salt stress, although less efficiently than glycine betaine. The presence of 1 mM choline in the growth medium conferred tolerance to high salinity on *A. halophytica* with the maintenance of high growth up to 1.5 M NaCl. The uptake of choline was $Na^+$-dependent, sensitive to various metabolic inhibitors as well as thiol-reactive agents. The results of competition studies suggested that *N*-methyl on one end of molecule and on the other end either an aldehyde, an alcohol or a neutral group were important features for substrate recognition.


*Keywords:* Choline uptake; Salt stress; Cyanobacteria

## 1. Introduction

High salinity in soils and in aqueous environment is an important physical factor that affects growth and survival of living organisms. In bacteria, salt stress can trigger the flux of water across the cytoplasmic membrane. Thus, to avoid dehydration under high salinity or lysis under low salinity growth conditions, bacteria must posses active mechanisms that allow them to adapt to changes in the concentration of salt in the environment [1,2]. Among these mechanisms, one of the most clearly established systems is the accumulation of organic compatible solutes or osmoprotectants like amino acids, sugars and betaines under conditions of high concentration of salt [2]. Such accumulations increase the cytoplasmic osmolarity without disturbing cellular metabolism [3]. The most universally adopted compatible solute is glycine betaine, which can be accumulated during salt stress or osmotic stress by a large variety of bacteria including cyanobacteria [4–6].

For the cyanobacteria, the type of compatible solutes synthesized or accumulated in the cells has been used to differentiate the degree of tolerance to external salinity. For example, strains with low salt tolerance (max 0.7 M) synthesize sucrose or trehalose, strains with moderate salt tolerance (max 1.8 M) synthesize glucosylglycerol and strains with high salt tolerance synthesize glycine betaine [7].

The accumulation of glycine betaine occurs either via transport from the environment or via biosynthesis from a two-step oxidation of choline with betaine aldehyde as an intermediate [4,6,8]. Choline itself can serve as an osmoprotectant in an organism provided that the organism possesses both choline uptake and choline oxidation activity. Mutants of *Escherichia coli* defective in their ability to convert choline to glycine betaine cannot grow at elevated osmotic strength [8]. The uptake of choline followed by two-step oxidation to glycine betaine is therefore an important element to confer tolerance to osmotic stress in microorganisms. The uptake of choline has been studied in a large

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number of bacteria [9–11] but so far no such study has been reported for cyanobacteria. Most of studies for the uptake of compatible solutes in cyanobacteria were done by Hagemann's group using a moderately halotolerant *Synechocystis* sp. PCC 6803, which accumulates glucosylglycerol in response to salt stress [12,13].

*Aphanothece halophytica* is a high salt tolerant cyanobacterium capable of growth in media containing NaCl concentration as high as 3.0 M [14]. The natural habitat of *A. halophytica* used in the present study is in Solar Lake, Israel. Previously we have shown that exogenously provided choline could confer salt tolerance on *A. halophytica* via the accumulation of glycine betaine [6]. Glycine betaine transport has been shown to be increased by *A. halophytica* grown in media of elevated osmotic strength [15]. This paper describes the influence of salt stress on choline uptake in *A. halophytica*. Evidence for the existence of a periplasmic choline binding protein is also presented for the first time in cyanobacteria.

## 2. Materials and methods

### 2.1. Chemicals

[*methyl*-$^{14}$C]Choline (58 mCi/mmol) was obtained from the Radiochemical Centre, Amersham, England. Dinitrophenol and potassium cyanide were obtained from E. Merck AG, Darmstadt, Germany. Acetylcholine, betaine aldehyde, chloramphenicol, *N*-ethylmaleimide, glycine betaine, gramicidin D, phosphorylcholine and sodium *p*-chloromercuribenzoate were purchased from Sigma Chemical Co., St. Louis, USA. Sodium arsenate, sodium fluoride, sorbitol and glycine were obtained from BDH, England. All reagents used were of analytical grade.

### 2.2. Growth conditions for A. halophytica

*A. halophytica* cells were grown photoautotrophically in $BG_{11}$ medium supplemented with 18 mM $NaNO_3$ and Turk Island salt solution as described previously at an irradiation of 60 $\mu Em^{-2}\ s^{-1}$ [16]. Cotton-plugged 250-ml conical flasks containing 100 ml of medium were used and shaken on a rotary shaker at 30 °C. The concentration of NaCl in the growth medium was adjusted by adding an appropriate amount of NaCl to the medium, i.e. 0.5 M for unstress and 2.0 M for salt-stress conditions. Cell growth was followed by monitoring $OD_{750}$.

### 2.3. Transport assays

Cells at late log phase were harvested by centrifugation (8000 × *g*, 10 min), washed twice with 50 mM Hepes–NaOH buffer pH 7.5, and suspended to a concentration of ca. 0.1 mg cell protein/ml in the same buffer containing either 0.5 M NaCl (unstress) or 2.0 M NaCl (salt-stress). The uptake experiment was initiated by adding [*methyl*-$^{14}$C]choline with a specific activity of 0.1 μCi/μmol at a final concentration of 50 μM or otherwise stated. The cell suspension was incubated at 37 °C with shaking at 200 rpm. Aliquots were withdrawn at 1-min intervals and rapidly filtered through HAWP cellulose nitrate filters (0.45-μm pore size; Millipore). The filters were washed twice with 3 ml of buffer (same osmolarity as the assay buffer) and the radioactivity trapped in the cells was determined by counting with a scintillation counter. The rate of choline uptake was linear for at least 3 min depending on cell density and choline concentration. Initial choline uptake rates were determined from the linear increase of uptake and are expressed as nanomoles of choline taken up per minute per milligram protein as determined by the method of Bradford [17] using bovine serum albumin as a standard. In inhibitory assays, the cells were preincubated with the inhibitor for 30 min at 37 °C before the addition of [*methyl*-$^{14}$C]choline. In competition experiments, cells were added to a mixture of labeled substrate and unlabeled analogs. Unless otherwise indicated, all data are averages of duplicate assays of two independent experiments whose values differ less than 10%.

### 2.4. Assay of periplasmic binding protein

Cells at late log phase grown either under normal growth conditions (0.5 M NaCl) or salt-stress conditions (2 M NaCl) were collected by centrifugation (8000 × *g*, 10 min) and washed twice with 10 mM Tris–HCl, pH 7.6 containing either 0.5 M or 2.0 M NaCl. Periplasmic proteins were released by osmotic shock according to Neu and Heppel [18]. Cells were resuspended in 20 ml of plasmolysis buffer containing 10 mM Tris–HCl pH 7.6, 0.5 or 2.0 M NaCl, 1.0 M sorbitol and 1 mM EDTA and shaken gently for 30 min at room temperature. Cells collected after centrifugation were resuspended in 10 ml of cold deionized water, frozen at −80 °C for 30 min and thawed at 37 °C for 30 min. After centrifugation the supernatant was centrifuged once more to remove remaining cells and used as periplasmic proteins fraction. Choline binding activity was detected by non-denaturing polyacrylamide gel electrophoresis (12%) using 50-μg periplasmic protein mixed with 10 μM [*methyl*-$^{14}$C]choline with an incubation at 20 °C for 30 min. The gels were quickly dried on Whatman 3 MM paper and autoradiographed with X-OMAT S Kodak films during 14 days.

## 3. Results

### 3.1. Protection against growth inhibition at high salt concentration by choline and glycine betaine

Growth of *A. halophytica* was retarded in the medium containing 2.0 M NaCl when compared to that containing 0.5 M NaCl (Fig. 1). The addition of either 1 mM choline

number of bacteria [9–11] but so far no such study has been reported for cyanobacteria. Most of studies for the uptake of compatible solutes in cyanobacteria were done by Hagemann's group using a moderately halotolerant *Synechocystis* sp. PCC 6803, which accumulates glucosylglycerol in response to salt stress [12,13].

*Aphanothece halophytica* is a high salt tolerant cyanobacterium capable of growth in media containing NaCl concentration as high as 3.0 M [14]. The natural habitat of *A. halophytica* used in the present study is in Solar Lake, Israel. Previously we have shown that exogenously provided choline could confer salt tolerance on *A. halophytica* via the accumulation of glycine betaine [6]. Glycine betaine transport has been shown to be increased by *A. halophytica* grown in media of elevated osmotic strength [15]. This paper describes the influence of salt stress on choline uptake in *A. halophytica*. Evidence for the existence of a periplasmic choline binding protein is also presented for the first time in cyanobacteria.

## 2. Materials and methods

### 2.1. Chemicals

[*methyl*-$^{14}$C]Choline (58 mCi/mmol) was obtained from the Radiochemical Centre, Amersham, England. Dinitrophenol and potassium cyanide were obtained from E. Merck AG, Darmstadt, Germany. Acetylcholine, betaine aldehyde, chloramphenicol, *N*-ethylmaleimide, glycine betaine, gramicidin D, phosphorylcholine and sodium *p*-chloromercuribenzoate were purchased from Sigma Chemical Co., St. Louis, USA. Sodium arsenate, sodium fluoride, sorbitol and glycine were obtained from BDH, England. All reagents used were of analytical grade.

### 2.2. Growth conditions for A. halophytica

*A. halophytica* cells were grown photoautotrophically in $BG_{11}$ medium supplemented with 18 mM $NaNO_3$ and Turk Island salt solution as described previously at an irradiation of 60 $\mu Em^{-2}\ s^{-1}$ [16]. Cotton-plugged 250-ml conical flasks containing 100 ml of medium were used and shaken on a rotary shaker at 30 °C. The concentration of NaCl in the growth medium was adjusted by adding an appropriate amount of NaCl to the medium, i.e. 0.5 M for unstress and 2.0 M for salt-stress conditions. Cell growth was followed by monitoring $OD_{750}$.

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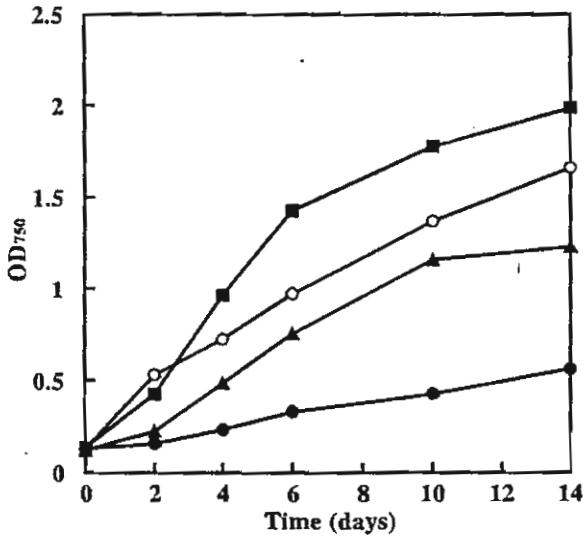


Fig. 1. Growth promoting effect of choline and glycine betaine on salt-stressed *A. halophytica*. Cells were grown under unstress condition, 0.5 M NaCl (○), under salt-stress condition 2.0 M NaCl (●), in 2.0 M NaCl plus 1 mM choline (▲), in 2.0 M NaCl plus 1 mM glycine betaine (■).

or glycine betaine alleviated the inhibitory effect. Glycine betaine showed a stronger protection than did choline. Growth under 2.0 M NaCl medium containing glycine betaine was even faster than that under normal growth conditions. Optimal stimulation of growth under salt stress was obtained at 1 mM choline and no stimulating effect was observed at higher than 10 mM choline (data not shown).

The growth rate of *A. halophytica* under different concentrations of NaCl with and without 1 mM choline is shown in Fig. 2. The growth rate was slightly stimulated by choline when NaCl was raised to 0.5 M. Growth of *A. halophytica* was inhibited at higher than 0.5 M NaCl in the absence of choline. The protective effect of choline against high salt concentration was observed when NaCl was increased up to 1.5 M. At higher than 1.5 M, the growth rate of *A. halophytica* decreased even in the presence of choline. Nevertheless, it is clear that up to 3 M NaCl the growth rates were higher in the presence than in the absence of choline.

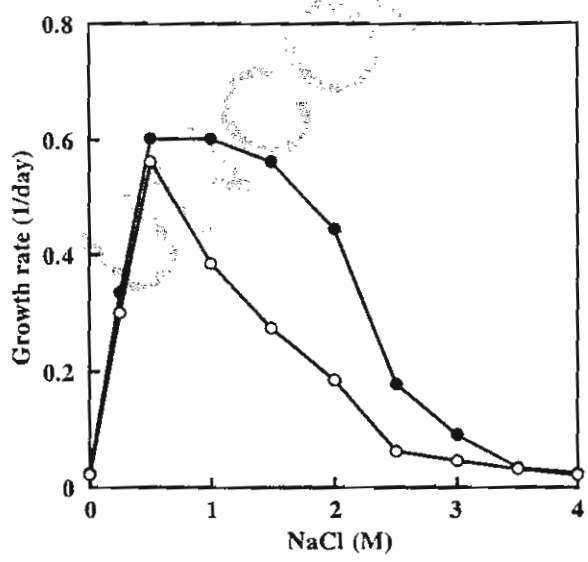


Fig. 2. Effect of choline on growth at high salinity. Cells were grown in media containing various concentrations of NaCl in the presence (●) or absence (○) of 1 mM choline. Growth rates were monitored by measuring $OD_{750}$ and were expressed as (doubling time)$^{-1}$.

### 3.2. Kinetics of choline uptake

Incubation of *A. halophytica* cells under unstress and salt-stress conditions with a choline concentration from 25 to 700 μM resulted in saturable initial uptake rates for both conditions (Fig. 3A). Double reciprocal plots yielded a straight line (Fig. 3B), indicating that the uptake follows typical

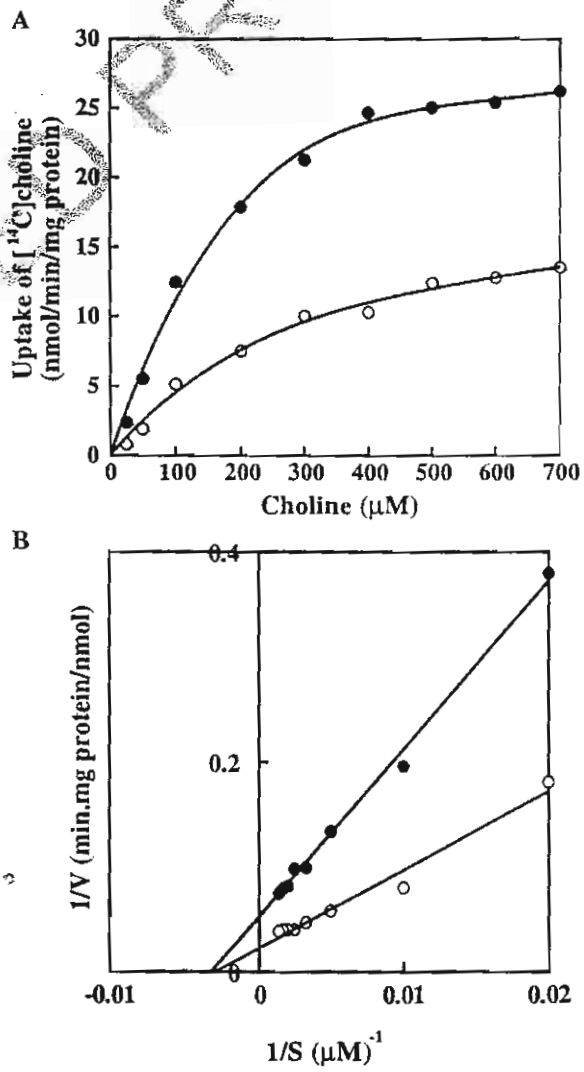


Fig. 3. Kinetics of choline uptake assayed under unstress condition, 0.5 M NaCl (○) or salt-stress condition, 2.0 M NaCl (●). (A) Substrate-saturable initial rates of choline uptake. (B) Lineweaver–Burk plot of the initial rates of choline uptake. The points shown are the means of three independent experiments, and the lines drawn are those derived from regression analysis of the data.

Michaelis–Menten kinetics. The line of best fit was performed by using a least-squares linear regression. The apparent affinity constant ($K_m$) values of 272 ± 24 and 286 ± 28 μM and the maximal velocity ($V_{max}$) values of 18 ± 1.5 and 37 ± 2.2 nmol/min/mg protein were obtained for unstressed and salt-stressed cells, respectively.

### 3.3. $Na^+$ dependence of choline uptake

In order to test whether salt stress affects choline uptake, the cells initially grown in 0.5 M NaCl containing medium were extensively washed with 50 mM Hepes–NaOH buffer pH 7.5 to get rid of residual NaCl. As shown in Fig. 4 choline uptake rates increased with increasing NaCl concentration in the assay medium. In the absence of NaCl, only marginal choline uptake was detected. In order to test whether an osmotic stress contributes to the increase of choline uptake, we measured choline uptake by incubating the cells with varying amounts of NaCl together with an appropriate amount of sorbitol so that the final osmolarity was equivalent to 4 osM. It was found that the rates of choline uptake were higher in cells with osmotic stress than those without osmotic stress, i.e. compare upper curve with lower curve of Fig. 4. Nevertheless, it should be noted that in the absence of $Na^+$, choline uptake for *A. halophytica* with osmotic stress was not different from that without osmotic stress. This indicates that the activity of choline uptake is not caused by an osmotic stress. Stimulatory effect of osmotic stress could be observed under the condition that adequate $Na^+$ was available, i.e. for *A. halophytica* higher than 50 mM $Na^+$ was needed (Fig. 4).

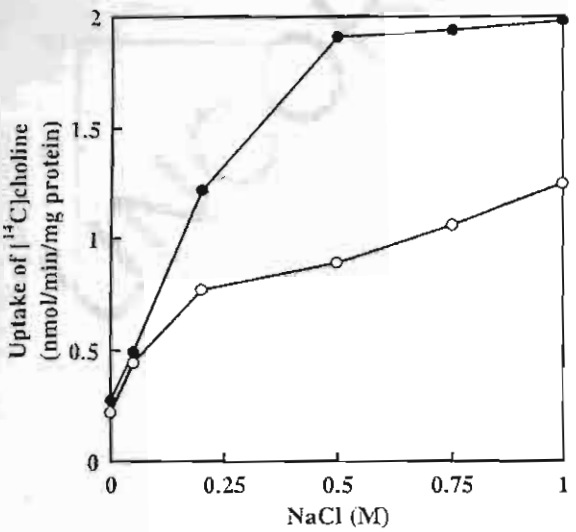


Fig. 4. Effect of different NaCl concentrations on choline uptake in the presence and absence of osmotic stress. Initial choline uptake rates were determined in the presence of various NaCl concentrations without osmotic stress (○) and with osmotic stress (●) in which the osmotic strength of the reaction mixture was kept constant at 4 osM by adding appropriate concentrations of sorbitol.

Table 1
Inhibition of choline uptake by unlabeled analogs[a]

| Compound | Inhibition (%) | $K_i$ (μM)[b] |
|---|---|---|
| Acetylcholine | 78 | 118 |
| Phosphorylcholine | 21 | 329 |
| Betaine aldehyde | 88 | 101 |
| Glycine betaine | 17 | 365 |
| Glycine | 18 | 353 |

[a] *A. halophytica* was grown in normal 0.5 M NaCl. Cells were incubated for 3 min in a mixture containing 50 μM [methyl-$^{14}$C]choline and 5 mM unlabeled inhibitor. Data are given as the percent inhibition of the uninhibited uptake rate which was 1.3 nmol/min/mg protein.

[b] Inhibition constant ($K_i$) was determined from measurements at four concentrations of substrate and four concentrations of inhibitor. Each value is the mean of three independent experiments (SE was within 9%).

### 3.4. Specificity of choline uptake

The specificity of choline uptake in *A. halophytica* was studied by addition of 100-fold excess of various compounds into the assay medium and following their initial choline uptake rates (Table 1). The choline analog, acetylcholine, acted as an effective competitor for choline whereas phosphorylcholine did not. Betaine aldehyde, differing from choline by having an aldehyde-group instead of an alcohol group, showed strong inhibition of choline uptake. However, glycine betaine and glycine with a carboxyl group were poor competitors for choline uptake.

We further assessed the transporter specificity through competitive inhibition by measuring the choline uptake rates at various substrate and inhibitor concentrations. The plot between inhibitor concentrations and the slopes obtained from double reciprocal plots yielded the approximate value of inhibition constant ($K_i$) as shown in Table 1. It was evident that acetylcholine and betaine aldehyde with strong inhibition had high affinity to the transporter. In contrast, phosphorylcholine, glycine betaine and glycine had low affinity to the transporter which subsequently led to weak inhibition.

### 3.5. Effect of various inhibitors on choline uptake

*N*-Ethylmaleimide and sodium *p*-chloromercuribenzoate which modify the protein structures were effective inhibitors of choline uptake (Table 2). The inhibitors for ATP formation, sodium arsenate and sodium fluoride also reduced choline uptake, although sodium fluoride was less efficient. The interference of the electron transport by potassium cyanide and of the generation of proton motive force by dinitrophenol resulted in effective inhibition of choline uptake. Gramicidin D that collapses $Na^+$ gradients also showed considerable inhibition of choline uptake.

### 3.6. Induction of choline transport by salt stress

Because salt stress led to an increase in initial choline transport rate (Fig. 4), we therefore investigated whether

Table 2
Effect of metabolic inhibitors on the initial rate of choline uptake[a]

| Inhibitor | Concentration | Percent inhibition |
|---|---|---|
| *N*-Ethylmaleimide | 0.50 mM | 97 |
| Sodium *p*-chloromercuribenzoate | 0.50 mM | 94 |
| Sodium arsenate | 1.00 mM | 95 |
| Sodium fluoride | 1.00 mM | 63 |
| Potassium cyanide | 1.00 mM | 83 |
| Dinitrophenol | 1.00 mM | 79 |
| Gramicidin D | 1.00 μg/ml | 74 |

[a] *A. halophytica* was grown for 30 min in normal 0.5 M NaCl. Cells were preincubated for 30 min in the presence of the indicated inhibitor before the addition of 50 μM [methyl-$^{14}$C]choline. Data are given as the percent inhibition of the uninhibited uptake rate which was 1.3 nmol/min/mg protein.

protein synthesis was involved in the stimulation of choline transport by salt stress. Choline transport was faster in stressed cells than in unstressed cells (Fig. 5). However, preincubation of the stressed cells with chloramphenicol (100 μg/ml) resulted in a significant decrease of choline transport. This suggests that the stimulation of transport by salt stress observed in the absence of chloramphenicol appeared to be dependent on de novo protein synthesis of either a transport protein or protein(s) regulating the activity of preexisting transport protein(s). Choline transport of unstressed cells in the presence of chloramphenicol was not significantly different from that in the absence of chloramphenicol, suggesting that the constitutive choline uptake was not affected by chloramphenicol. The observation that the increased choline transport was not completely abolished in the chloramphenicol-treated cells under salt stress could be ascribed to the possibility that chloramphenicol used at the concentration of 100 μg/ml could not completely inhibit protein synthesis. Indeed we found that virtually no choline transport occurred when cells were treated with 500 μg/ml chloramphenicol (data not shown).

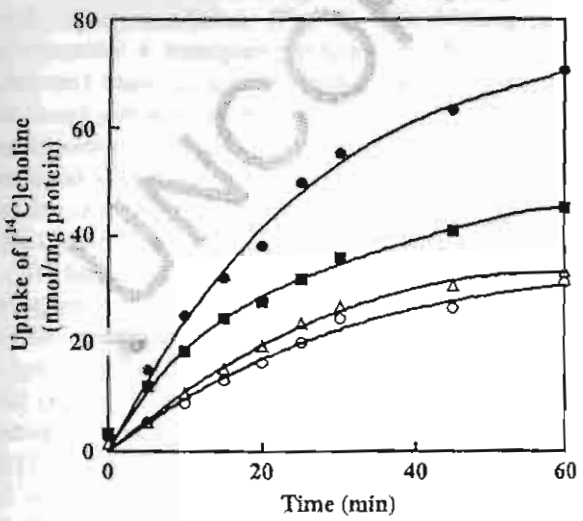


Fig. 5. Effect of chloramphenicol on choline uptake. Choline uptake was assayed under unstress condition, 0.5 M NaCl (○), under salt-stress condition, 2.0 M NaCl (●), in 0.5 M NaCl plus 100 μg/ml chloramphenicol (△), in 2.0 M NaCl plus 100 μg/ml chloramphenicol (■). In all cases, preincubation for 30 min prior to the addition of labeled choline to initiate the uptake was done.

### 3.7. Evidence for periplasmic choline binding protein

To analyze the existence of a choline binding protein, periplasmic fractions from unstressed and salt-stressed cells were subjected to nondenaturing polyacrylamide gel electrophoresis in the presence of [*methy*-$^{14}$C]choline. One single radioactive band was detected for cells under unstress and salt-stress conditions with more intense band observed for the latter (Fig. 6; lanes 2 and 3). The sample containing only [*methy*-$^{14}$C]choline but without periplasmic protein showed no band (Fig. 6; lane 1).

To verify that the periplasmic fractions obtained by cold osmotic shock used in this study contained periplasmic proteins and not cytoplasmic proteins, we checked for the marker enzyme activities [19]. With identical amounts of protein used, high activity of alkaline phosphatase was detected in the periplasmic fractions whereas none was detected in the total protein fractions. On the other hand, the specific activity of isocitric dehydrogenase which is a cytoplasmic marker enzyme accounted for less than 7% of that by the total protein fractions. These results demonstrated that the periplasmic fractions were not contaminated with cytoplasmic proteins. Furthermore, we also checked

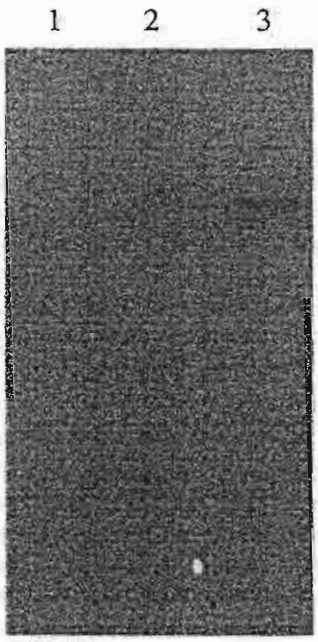


Fig. 6. Existence of choline binding protein from periplasmic fluid as revealed by autoradiography of nondenaturing gel electrophoresis. Lane 1 is sample without periplasmic proteins. Lanes 2 and 3 are samples of periplasmic proteins from cells grown under unstress (0.5 M NaCl) and salt-stress (2.0 M NaCl) conditions, respectively.

whether the cells depleted of periplasmic fractions were able to take up choline. Only low level of $^{14}$C-choline could be detected in the periplasm-depleted cells after 30-min incubation, representing less than 5% of that by intact cells. This indicates that a choline binding protein was required for choline uptake and it was localized in periplasmic fractions.

## 4. Discussion

The results presented in this study clearly demonstrate the existence of a transport system for choline in *A. halophytica*. Exogenously provided choline could be transported into *A. halophytica* and could serve as a growth promoting substance when the cells were grown at high external salinity (Figs. 1 and 2). The protective role by choline against inhibition of growth at high salinity was not as efficient as that by glycine betaine, suggesting that choline per se is not an osmoprotectant. This was supported by our earlier observation that the transported choline was efficiently converted to betaine aldehyde and finally to glycine betaine in *A. halophytica* under high salinity [6]. Glycine betaine is also shown to be more efficient than choline in the stimulation of growth of the moderate halophilic bacterium *Halomonas elongata* over the range of salinity from 0.5 to 2.0 M NaCl [20].

The transport system for choline in *A. halophytica* is an active transport. This conclusion is based on the following three lines of evidence. First, the kinetic data indicate that choline transport is substrate-saturable and shows a Michaelis–Menten relationship (Fig. 3A), observations which support the involvement of a transport protein. Second, the existence of a periplasmic choline binding protein was demonstrated with increasing content in cells grown under high salinity conditions (Fig. 6). This binding protein is presumably a transport protein for choline since the increased transport activity under salt stress is due to an increased synthesis of transport protein (Fig. 5) rather than an activation of the existing transport protein. Third, choline transport is highly inhibited by various energy generation inhibitors including inhibitors, which modify protein structure (Table 2).

We have shown here that $Na^+$ was required for optimum transport activity of choline as only marginal level of choline uptake could be detected in the absence of $Na^+$, regardless of whether the cells were under osmotic stress or not (Fig. 4). Specific requirements of $Na^+$ for transport activities have been well documented in marine bacteria [21]. Very recently we have also shown that nitrate uptake in *A. halophytica* is dependent on $Na^+$ [22]. An increase of NaCl concentration resulted in higher choline uptake rate in *A. halophytica*. It seems likely that the rate of transport was affected by the concentration of NaCl providing $Na^+$ as a coupling ion for transport and $Na^+$ and $Cl^-$ as sources of osmotic stress. In view of the fact that osmotic stress alone without $Na^+$ caused only modest choline uptake (Fig. 4), it is likely that the primary role of $Na^+$ is to act as a coupling ion. The result showing the inhibition of choline transport by gramicidin D (Table 2), a reagent which collapses $Na^+$-gradients, suggests an involvement of $Na^+$-gradients in the transport of choline by *A. halophytica*. In addition, dinitrophenol, an uncoupler disrupting the proton motive force, was a potent inhibitor of choline transport. Taken together, it may be that an $Na^+$-gradient is created in exchange for the proton gradient. Indeed, *A. halophytica* has recently been shown to contain an $Na^+/H^+$ antiporter with a major role for salt stress protection [23].

Previously, $Na^+$-activated glycine betaine transport in *A. halophytica* has been reported [15]. As *A. halophytica* is a halophilic cyanobacterium, its normal growth medium contains about 0.5 M NaCl. However, it can adapt to increasing external salinity by synthesis and accumulation of glycine betaine [6,24]. It is apparent that *A. halophytica* can rely on either the synthesis or the uptake of glycine betaine to osmoregulate against high external salinity. However, the latter process is preferable due to the fact that de novo synthesis is energetically more expensive than the transport process [25].

Choline transporter seems to be distinct from glycine betaine transporter in *A. halophytica* since glycine betaine was a poor competitor for choline transport (Table 1). It appears that *N*-methyl groups on one end of the molecule and an alcohol or aldehyde group on the other end are important for the affinity to the choline transporter. The charge of the molecule is also important in the recognition by the choline transporter because the most effective competitors are positively charged (acetylcholine, betaine aldehyde), whereas zwitterionic compounds closely related to choline (phosphorylcholine, glycine betaine) are less effective.

The data in Fig. 3B revealed that the choline transport system of *A. halophytica* had a low affinity for choline ($K_m$, 272 μM) which is in contrast to a rather high affinity for glycine betaine transporter ($K_m$, 2 μM) [15]. The levels of choline have been estimated to be in nanomolar range in coastal seawater [26]. The data on the levels of glycine betaine in seawater were not available. In general, the amount of glycine betaine available in the environments depends on many factors including the level of organic material, the rate of microbial degradation of dead cells and the atmospheric conditions. Presuming that the level of glycine betaine in seawater is very low, it is therefore likely that the functional glycine betaine transporter rather than choline transporter may play a role to allow *A. halophytica* to thrive in coastal seawater. However, choline transport in *A. halophytica* could clearly protect cells against high salinity stress provided that sufficient concentration of choline is available as shown in Fig. 2. There remains a possibility that the physiological role of this transporter is to transport choline for the supply of carbon and nitrogen sources under certain conditions. *Corynebacterium glutamicum*, a gram-positive soil bacterium, has previously been

shown to contain a specific proline carrier, Put P, which is not involved in osmoregulation but is responsible for proline utilization [27]. This Put P carrier also showed low affinity for proline, a similar property with respect to low affinity for choline by choline transporter in *A. halophytica*.

The present study seems to suggest that *A. halophytica* possesses only one transport system for choline with low affinity. However, kinetic data alone cannot conclusively exclude the possible multiple choline transport systems. Previously two or more choline transport systems have been found in *Pseudomonas aeruginosa* [28], *Rhizobium meliloti* [29] and *Bacillus subtilis* [30]. The fact that choline binding protein in the periplasmic fraction could be osmotically induced (Fig. 6) suggests that the transporter involved in choline uptake is an ATP-binding cassette (ABC) transporter. Since this kind of transporter usually has high affinity for its substrate, it is difficult to reconcile with the low affinity for choline suggested by kinetic data of the present study. However, recently Jebbar et al. [31] reported that ectoine was taken up by *B. subtilis* via the ABC-transport system Opu C with low affinity ($K_m$ = 1.5 mM). Furthermore, another ABC-transport system Pro U in *E. coli* was also able to transport ectoine with low affinity ($K_m$ = 200 μM) and this transport of ectoine appeared to involve a periplasmic binding protein [32].

At present it is unclear how *A. halophytica* with one system of low affinity choline transport can cope with high salinity environments. Worth mentioning in this regard is the fact that *A. halophytica* is able to synthesize glycine betaine de novo [24]. Hyperosmotic stress led to an increased accumulation of glycine betaine in *A. halophytica* without exogenous supply of choline. This suggests that choline-glycine betaine pathway might not significantly contribute to osmoregulation by *A. halophytica*. Although we previously showed that exogenously provided choline led to an increase of glycine betaine under hyperosmotic stress [6], we speculate that *A. halophytica* might as well synthesize glycine betaine via a three-step series of methylation reactions from glycine. This mechanism of glycine betaine synthesis has been reported in a number of bacteria and very recently in extreme halophiles [33,34]. The physiological role of choline transport of *A. halophytica* in natural habitats is likely to import choline as carbon and nitrogen sources. However, when high concentration of external choline is available, it can be taken up by low-affinity choline transporter and finally converted to glycine betaine for osmoregulatory function.

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